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Low oxygen affects photophysiology and the level of expression of two carbon metabolism genes in the seagrass *Zostera muelleri*.

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ABSTRACT

Seagrasses are a diverse group of angiosperms that evolved to live in shallow coastal waters, an environment regularly subjected to changes in oxygen, carbon dioxide and irradiance. *Zostera muelleri* is the dominant species in south-eastern Australia, and is critical for healthy coastal ecosystems. Despite its ecological importance, little is known about the pathways of carbon fixation in *Z. muelleri* and their regulation in response to environmental changes. In this study, the response of *Z. muelleri* exposed to control and very low oxygen conditions was investigated by using (i) oxygen microsensors combined with a custom-made flow chamber to measure changes in photosynthesis and respiration, and (ii) Reverse Transcription quantitative real-time PCR (RT-qPCR) to measure changes in expression levels of key genes involved in C_4 metabolism. We found that very low levels of oxygen (i) altered the photophysiology of *Z. muelleri*, a characteristic of C_3 mechanism of carbon assimilation, and (ii) decreased the

expression levels of phosphoenolpyruvate carboxylase (PEPC) and carbonic anhydrase (CA). These molecular-physiological results suggest that regulation of the photophysiology of *Z. muelleri* might involve a close integration between the C_3 and C_4, or other CO_2 concentrating mechanisms metabolic pathways. Overall, this study highlights that the photophysiological response of *Z. muelleri* to changing oxygen in water is capable of rapid acclimation and the dynamic modulation of pathways should be considered when
assessing seagrass primary production.

INTRODUCTION

Seagrasses are a diverse group of monocotyledonous angiosperms that evolved to live in the marine environment during the Cretaceous period, approximately 100 million years ago (Larkum and den Hartog 1989). There are approximately 72 seagrass species in 12 genera worldwide (Short et al. 2011) playing an important role in coastal ecosystems (Costanza et al. 1997). Indeed, highly productive seagrass ecosystems provide food and shelter for commercially important fish (Beck et al. 2001) with temperate seagrass meadows in southern Australia estimated to supply onshore fisheries valued at \$A 230 000 ha^{-1} y^{-1} (Blandon and Zu Ermgassen 2014), and enhance sediment accretion (Koch et al. 2013). Seagrasses have also recently been identified as a major carbon sink, responsible for 10-18% of the Ocean's carbon accumulation (McLeod et al. 2011; Fourqurean et al. 2012; Greiner et al. 2013).

Many seagrasses are intertidal species which grow in shallow coastal lagoons and are therefore exposed to large variations in light and sediment loading/resuspension (Harlin 1995). Additionally, seagrasses are exposed to large fluctuations in oxygen levels, ranging from 71 to 311 µmol L^{-1} under normal conditions (Brodersen et al. 2017) and as low as 10% (approx. 20 µmol L^{-1}) air saturation during night time in areas where seagrass die-offs were observed (Borum et al. 2005). They also have anatomical adaptations such as the absence of stomata and the development of extensive aerenchyma (Penhale and Wetzel 1983) along with physiological

adaptations such as the ability to tolerate hypoxic and anoxic conditions especially in the roots and rhizomes (Pregnall et al. 1984; Papenbrock 2012) which they possibly inherited from submerged freshwater ancestors (Les et al. 1997). As seagrass persistence generally require a large flux of photosynthetically active radiation, roughly 10% of surface irradiance (Papenbrock 2012), the effects of light on seagrass ecology have been extensively studied (Ralph et al. 2007; Staehr and Borum 2011; Brodersen et al. 2015;
Chartrand et al. 2016). However, less attention has been given to the effects of photosynthetic gases and associated metabolic pathways in seagrasses, e.g., the effects of low O_2 conditions have only been reported in a few papers to our knowledge (e.g. Black et al. 1976; Downton et al. 1976; Beer et al. 2002; Greve et al. 2003; Buapet et al. 2013).

The photosynthetic processes of seagrasses are very similar to that of other angiosperms (Beer et al. 1998). Most seagrasses were classified biochemically as C_3 plants on the biochemical criteria of short-term inorganic ^{14}C incorporation products and the ratio of Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) to phosphoenolpyruvate carboxylase (PEPC) activities, although *Thalassia testudinum* has C_4 metabolism (Benedict and Scott 1976) and *Halophila stipulacea* has C_3-C_4 intermediate metabolism by these criteria (Beer et al. 1980; Beer et al. 2002; Koch et al. 2013). The C_3 CO_2 fixation process begins with RuBisCO which catalyses the carboxylation using CO_2 (and H_2O) of ribulose 1, 5-bisphosphate (RuBP), producing two of the 3 carbon molecules; 3-phosphoglycerate (PGA) as the initial stable product. In parallel there is the oxygenation of RuBP with O_2 to yield one PGA and one 2-phosphoglycolate. The ratio of carboxylase to oxygenase activity is determined by the kinetic properties of the molecular form of RuBisCO involved and the CO_2:O_2 ratio at the active site of RuBisCO.

The C_4 carbon fixation process on the other hand, begins with the carboxylation (using HCO_3^- as the immediate inorganic C substrate) of phosphoenolpyruvate (PEP) in the RuBisCO-free cytosol, forming the 4-carbon acid oxaloacetate as the initial stable product. Oxaloacetate is subsequently converted to malate and/or aspartate which are moved to, and decarboxylated in, the compartment containing RuBisCO, generating CO_2 which is used in the carboxylation of RuBisCO, and a 3-carbon acid that returns to the cytosol, regenerating PEP. C_4 photosynthesis involves a higher steady-state CO_2 concentration than that available to RuBisCO in biochemically and physiologically defined C_3 photosynthesis with diffusive entry of CO_2 from the bulk external medium to RuBisCO, i.e., C_4 photosynthesis acts as a CO_2 concentrating mechanism (CCM). The regeneration of PEP to allow further PEPC activity is an energy (as ATP) requiring process. The C_4 mechanism has an advantage over the C_3 mechanism in low CO_2 and/or high O_2 environments as PEPC reacts specifically with CO_2 (after its conversion to HCO_3^-) with no interference from O_2 and with accumulation of CO_2 around RuBisCO that largely suppresses RuBisCO oxygenase activity. In C_3 plants, to a much smaller extent, the 2-phosphoglycolate from the oxygenase reaction is metabolised to

PGA and then sugar phosphates in energy-requiring photorespiratory carbon oxidation cycle (PCOC); whether the oxygenase-PCOC combination physiologically decreases the energetic efficiency of C_3 plants relative to C_4 plants depends on the energy cost of oxygenase-PCOC relative to the cost of operating the C_4 pathway with its inevitable leakage of CO_2 from the pool accumulated around RuBisCO (Raven 2014; Raven and Beardall 2016; Larkum et al. 2017). It is important to note that the occurrence of any CCM gives physiological (not biochemical) characteristics similar to those of a C_4 plant (Raven 2014; Raven and Beardall 2016, and references therein; Larkum et al. 2017). Thus, photosynthesis that is relatively insensitive to O_2, has a high affinity for CO_2 and can deplete the CO_2 in a closed system to very low

concentrations and is not diagnostic of C_4 photosynthesis since it could also result from any CCM activity.

In marine plants, inorganic carbon species diffusion to the leaf surface has a greater potential to restrict the rate of photosynthesis due to the slower diffusion rates in water. Further, the primary form of dissolved inorganic carbon in seawater is HCO_3^- (90%) with CO_2 making up about 1% (see below). Hence a CCM would be advantageous for marine plants, for example, physiologically "C_4-like" inorganic carbon acquisition has been observed in the seagrass *Zostera noltii* based on high light saturation values and a lack of observable photorespiration (Raven 1984; Jiménez et al. 1987); however, as noted by Raven (1984), this is not diagnostic of C_4 rather than some other CCMs. By contrast Waghmode and Joshi (1983), using *Halophila beccarii* (as *H. beccaeii*), showed short-term inorganic ^{14}C labelling of aspartate, and also alanine, i.e. features of C_4 photosynthesis, although critics could say that the labelling period was not short enough to show the real initial product of inorganic C assimilation. Despite this, short-term inorganic ^{14}C labelling is the most conclusive methods to indicate C_4 photosynthesis in seagrasses. The high ratio of PGA phosphatase to 2phosphoglycolate phosphatase activity in *H. beccarii* (Waghmode and Joshi 1983) is consistent with a decreased RuBisCO oxygenase generating 2-phosphoglycolate, and hence decreased flux through the PCOC producing glycine and serine, and a requirement for the non-phosphorylated pathway from photosynthetic or glycolytic PGA to glycerate and hence to serine and glycine. However, the decreased RuBisCO oxygenase and concomitant requirement for the PGA to glycerate pathway to serine and glycine could occur in any organism with a CCM. Colman and Norman (1997) showed the occurrence of a phosphorylated pathway, not involving PGA phosphatase, from PGA to serine in cyanobacteria with CCMs and minimal 2-phosphoglycolate synthesis. The occurrence of PEPC and aspartate aminotransferase in *H. beccarii* does not signify a C_4 pathway, since these enzymes are ubiquitous in plants (Aubry et al. 2011). HCO_3^- is the predominant inorganic C

species in seawater (at pH 8.16: 85.8% HCO_3^-, 0.4% CO_2 and 13.7% CO_3^{2-}, Pierrot et al. 2006) with CO_2 at about the same concentration (mol $m^{-3}$of fluid medium) as in air. However, the diffusion coefficient for CO_2 in water is about 10^{-4} that in air (Raven 1984) so, despite the usually thinner diffusion boundary layer in water (~0.01-0.1 mm) than in air (~1 mm) under ecologically relevant conditions (Vogel 1994), CO_2 diffusion to the leaf surface, and O_2 diffusion from the leaf surface (Mass et al. 2010) may limit photosynthesis in marine plants more than in land plants. Although the diffusion coefficient for HCO_3^- is lower than that of CO_2 (Raven 1984), the quantitative predominance of HCO_3^- in seawater means that it can support a larger flux to the leaf surface in response to a given potential for CO_2 assimilation in the leaf, provided the leaf can use HCO_3^-. Some seagrasses can utilise HCO_3^- either directly via active transport into epidermal cells (Beer and Rehnberg 1997), or more commonly, indirectly by dehydrating HCO_3^- to CO_2 via the enzyme carbonic anhydrase (CA) in the epidermal cell wall (Beer et al. 1980) usually interacting with co-localised leaf surface acidification by energy-requiring H^+ efflux (Hellblom et al. 2001; Hellblom and Axelsson 2003; Borum et al. 2016). Such indirect methods for enhancing inorganic C (Ci) uptake make predictions of C_4 mechanisms in seagrasses doubtful unless supported by strong evidence.

The suggestion that C_4 photosynthesis does not occur in seagrasses because of the absence of bundle sheath cells containing chloroplasts and the lack of true Kranz anatomy (Beer et al. 1980) has subsequently been shown to be invalid. Some freshwater submerged aquatic plants and some terrestrial C_4 members of the Chenopodiaceae utilise C_4 photosynthesis via the fixation of external inorganic C by PEPC, and the fixation of CO_2 (regenerated from C_4 acid decarboxylation) by RuBisCO, the carboxylases occurring in the cytosol and the chloroplasts respectively of a single cell (Voznesenskaya et al. 2001; references in Raven and Beardall 2016).

With recent advances in genomics and transcriptomics, researchers have the capacity to examine molecular mechanisms which drive seagrass photosynthesis to an extent that was unimaginable just a decade ago. In this context, the presence of genes encoding enzymes characteristic of the C_4 carbon fixation pathway in seagrass transcriptome could provide evidence relevant to the argument that seagrasses are not strictly C_3 plants.

For instance, several genes coding for PEPC, a cytosolic enzyme essential for the C_4 carbon fixation pathway in higher plants (Chollet et al. 1996), have been identified in the transcriptome of *Zostera muelleri* (unpublished data). This enzyme catalyses the irreversible β-carboxylation of phosphoenolpyruvate (PEP) by HCO_3^- to produce oxaloacetate (as described previously), a key intermediate in the C_4 carbon fixation pathway. However, PEPC has a ubiquitous anaplerotic role in plants and algae (excluding dinoflagellates where PEPC is replaced by pyruvate carboxylase) in replenishing the intermediates of the Krebs cycle depleted by the use of oxaloacetate and 2-oxoglutarate in the synthesis of some amino acids and of pyrimidines haems and chlorins (Raven 1984; Raven and Farquhar 1990; Aubry et al. 2011; Raven 2014). Additional PEPC expression is needed in the roots of seagrasses growing on carbonate substrata in the production of organic acids that release phosphate from apatite in the carbonate sediment (Long et al. 2008; Raven 2014). Chi et al. (2014) show that there is at least 1 copy of each of 8 genes related to C_4 photosynthesis and also to other aspects of metabolism in the 4 completely sequenced tracheophytes (2 with C_3 photosynthesis, 2 with C_4 photosynthesis) and 1 completely sequenced C photosynthesis bryophyte. For PEPC, the 2 C_4 plants had 3 or 4 copies of the gene, while the 2 C_3 plants have 4 or 6 copies. Therefore, the presence of the PEPC gene in the transcriptome of *Zostera muelleri* (unpublished data) does not show that *Z. muelleri* is other than, biochemically, a C_3 plant.

Furthermore, genes encoding CA were also detected in the *Z. muelleri* transcriptome

(unpublished data). CA catalyses the reversible interconversion of HCO_3^- to CO_2 ($HCO_3^- + H^+ = CO_2 + H_2O$). CAs are also involved in several non-photosynthetic reactions in plants (Raven 2014 and references therein), possibly including provision of respiratory CO_2 to HCO_3^- for the PEPC activity (Raven 2014) required for synthesis of the organic acids used, after secretion, in phosphate release from carbonate substrata (Long et al. 2008; Raven 2014) in seagrass roots. One or more CAs are components of C_4-based and other CCMs, as well as in C_3 photosynthesis (Aubry et al. 2011; Raven 2014; Raven and Beardall 2016).

However, expression of C_4 photosynthetic metabolism in some submerged freshwater relatives of seagrasses is a function of environmental conditions (low CO_2, high O_2), unlike terrestrial C_4 plants where it is constitutive (references in Raven and Beardall 2016).

Therefore it is possible that the expression of some PEPC and CA genes in seagrasses varies with the O_2 concentration and hence the potential for RuBisCO oxygenase activity, noting that the other light dependent O_2 consuming reactions, i.e. the water-water (or oxygenoxygen) cycles of the Mehler Peroxidase reaction and of the oxidation by the plastid terminal oxidase of plastoquinone reduced by PSII, are minimal in the only seagrass (*Zostera marina*) investigated: Buapet and Björk (2016).

The aim of this molecular-physiological study was to address the following: (i) how the photosynthetic and respiratory rates are affected by experimentally reduced O_2 concentration in the water column using electrochemical microsensors and (ii) how this reduced O_2 concentration affects the expression levels of PEPC and CA using Reverse Transcription quantitative real-time PCR (RT-qPCR).

MATERIAL AND METHODS

Seagrass collection and experimental setup

Specimens of *Zostera muelleri* ssp. *capricorni* (Asch) S. W. L. Jacobs and attached marine sediment were collected from Pittwater, NSW, Australia (33° 38’ 45.6”S, 151° 17’ 12.8”E) on the 14th of May 2015. In order to mimic the conditions of Pittwater at the University of Technology Sydney (UTS) aquarium facility, salinity and temperature of the water were measured in the field; ambient salinity: 31 and water temperature: 22°C, along with rapid light curves of *Z. muelleri* to determine suitable light conditions. Rapid light curves measured in the field on 3 *Z. muelleri* plants using a Diving-Pulse Amplitude Modulated (PAM) fluorimeter (DIVING-PAM, Heinz Walz GmbH, Eichenring, Germany) indicated that photosynthetic saturating light was approximately 230 µmol photons m^{-2} s^{-1}, which is

consistent with saturating light levels previously found for this seagrass species in temperate regions (Schwarz 2004; Bulmer et al. 2016). Collection was performed at low tide in shallow water (~1 m) and plants were transported immediately to an aquarium facility at the University of Technology Sydney. Before further handling, the specimens and sediment were placed into aquaria for 48 hours, after which they were separated into individual ramets/shoots (see Procaccini et al. 2007). These samples were then acclimated for 2 months in 40 L glass aquaria to conditions mirroring that of the sampling site e.g.: a salinity of 31, temperature of 22±1°C and illumination with an incident photon irradiance of ~230 µmol photons m^{-2} s^{-1} (12 h : 12 h light : dark cycle). Sixteen individual shoots of *Z. muelleri* were then transplanted in pairs into 8 plastic tanks (~150 mm in diameter, 4 tank replicates per treatment) with ~30 mm of sediment and acclimated at the same environmental conditions as above for 2 weeks.

The experiment was initiated in the middle of the photoperiod. Each of the treatments (i.e. control and low O_2) had one 100 L sump (100 L plastic bin) underneath a 40 L aquaria/table that held experimental plastic containers (Supplementary Figure S1). From each sump, water with corresponding O_2 level was pumped into four replicate plastic containers resulting in a total of 8 containers (4 replicate containers x 2 sumps/O_2 treatments = 8 containers in total). Each container contained 2 individual shoots of *Z. muelleri* (8 tanks x 2 shoots = 16 shoots). For the low O_2 treatment, the dissolved O_2 was lowered in the sump over an interval of 1 h via flushing with nitrogen gas (CO_2-free) to an average O_2 concentration of ~9 µmol O_2 L^{-1} as measured by a calibrated dissolved O_2 probe (FDO 925, WTW GmbH, Germany). The dissolved O_2 concentration within the aquaria was maintained between 9 and 16 µmol O_2 L^{-1} for the duration of the experimental period of 24 h (Supplementary Figure S2). Deviations from the initial pH of 8.16 (+/-0.01 pH) was controlled via bubbling of 99.9% pure CO_2, which was automatically controlled by a calibrated pH/CO_2 controller (7074/2, TUNZE Aquarientechnik GmbH, Germany, Supplementary Figure S3). The control tank set up was the same except for bubbling air instead of N_2 and CO_2.

Oxygen measurement setup

The lower half of leaf 2 (~20 mm) were cut from 3 individual ramets/shoots (see Procaccini et al. 2007) randomly picked from a pool of untreated samples which were previously subjected to the same acclimation procedure. The leaf sections were cleaned of any epiphytes and then fixed in place with fine pins on a piece of styrofoam in a custom-made flowchamber (see Brodersen et al. 2014) . The sections were angled in such a way as to allow for unobstructed flow over each of the sampling areas of the leaves. Illumination of the leaves to the desired light levels was achieved via a fibre-optic tungsten halogen lamp (KL-2500LCD,

Schott GmbH, Germany) with the irradiance measured at the leaf surface using a 4π quantum sensor (US-SQS/L, Walz GmbH, Germany) connected to a calibrated light meter (LI-250A,

LI-COR Inc., USA). Seawater was pumped through the flow chamber at a constant rate of ~5 mm s^{-1} for the duration of the experiment. Atmospheric air was bubbled during the control phase of the experiment, while nitrogen gas was bubbled during the treatment phase of the experiment, lowering the O_2 concentration from ~231 µmol O_2 L^{-1} to ~9 µmol O_2 L^{-1} (as described above). Salinity, temperature and pH of the seawater were kept constant throughout the experiment.

Vertical O_2 concentration micro-profiles towards the leaf tissue surface (approx. 0.031 cm^2) and thus across the diffusive boundary layer (DBL) were recorded using a Clark-type O_2 microsensor (OX-50, tip diameter approx. 50 µm; Unisense A/S Aarhus, Denmark; Revsbech, 1989) with a fast response time (<0.5 s) mounted on a motorized micromanipulator (Unisense A/S, Aarhus, Denmark). The microsensor was connected to a multimeter (Unisense Microsensor Multimeter A/S. Aarhus, Denmark) and interfaced with a PC running dedicated data acquisition and positioning software (SensorTrace PRO; Unisense A/S, Aarhus, Denmark). The microsensor was positioned at the leaf tissue surface (defined as 0 µm) manually by observing the microsensor tip and leaf tissue surface through a stereomicroscope mounted on an articulating arm. Subsequent measurements of vertical O_2 concentrations for micro-profiles were measured at 100 µm increments using the motorised micromanipulator (Unisense Motorised Micromanipulator A/S, Aarhus, Denmark) controlled by dedicated positioning software (SensorTrace PRO). Linear calibration of the O_2 microsensor was obtained from signal readings in 100% air-saturated seawater and anoxic seawater (seawater amended via N_2 bubbling and the O_2 scavenger sodium dithionite) at experimental salinity, pH and temperature.

Seagrass maximum quantum efficiency of photosystem II (Fv/Fm; Baker 2008) values were measured regularly on 3 biological replicates using a Pulse Amplitude Modulated (PAM) fluorimeter (Pocket PAM, Gademann Instruments, Wuerzburg, Germany, see Figueroa et al.

2013) after dark-adaptation for ~10 min. Minimal fluorescence (F_0) was recorded using a weak measuring light, which was then followed by a saturating pulse (irradiance of 3,500 µmol photons m^{-2} s^{-1} for 0.8 s) to measure maximal fluorescence (Fm). Under these conditions, Fv/Fm ratios provides a measure of maximal PSII photochemical efficiency ($Fv=Fm-F_0$) and were, in this experiment, used as an indicator of seagrass maximum quantum efficiency of PSII during experimentation.

Photosynthesis-Irradiance (P-I) curves

Established methods for determining rates of photosynthesis in marine plants via O_2 microsensors were used in this study (see Jørgensen and Revsbech 1985; Kühl et al. 1995; Lichtenberg and Kühl 2015; Pedersen et al. 2016). The effective DBL thickness was estimated by extrapolating the linear O_2 concentration gradient until it intersects with the constant O_2 concentration in the overlaying water. These O_2 micro-profiles determined at the leaf tissue surface in the flow chamber were measured at incident photon irradiances of 0, 25, 50, 100, 200, 500 and 700 µmol photons m^{-2} s^{-1}, first under control conditions (aerated seawater, 31 salinity, 22±1°C and pH 8.16). Leaves were then exposed to treatment conditions (~9 µmol O_2 L^{-1}, 31 salinity, 22±1°C and pH 8.16) at the beginning of the subsequent light cycle for 3 h before measurements were taken at incident photon irradiances in the following order: 25, 50, 100, 200, 500, 700 and 0 µmol photons m^{-2} s^{-1}. Leaves were allowed to equilibrate to each of the incident photon irradiances for 30 mins before O_2 microprofiles were recorded.

Based on the measured O_2 concentrations around the leaf tissue surface, O_2 fluxes were calculated using Fick's first law of diffusion:

(1) $J_{O_2} = -D_0 \frac{\Delta C}{\Delta z}$

where D_0 is the diffusion coefficient of O_2 in seawater at the experimental salinity and temperature (2.2088 x 10^{-5} cm^{-2} s^{-1}; tabulated values taken from **{** HYPERLINK

"http://www.unisense.com/" \h **}{** HYPERLINK "http://www.unisense.com/" \h **}** and $\frac{\Delta C}{\Delta z}$ is the gradient of the linear O_2 concentration within the DBL.

The measured O_2 fluxes across the leaf surface, which are the equivalent of rates of net photosynthesis, were then fitted with an exponential saturation model (Webb et al. 1974; Lichtenberg and Kühl 2015) using OriginPro (OriginLab, USA) with the added respiration term, *R*, to account for O_2 consumption (Spilling et al. 2010):

$$(2)\ P(E) = P_{max}\left(1 - exp^{\frac{-\alpha}{P_{max}}}\right) + R$$

where α is the initial slope of the P-I curve in the light-limiting phase, P_{max} is the maximum net photosynthetic rate and *R* is the respiration term.

This allowed for calculations of the minimum photosynthetic saturation irradiance (E_k), which gives an indication of the onset of photosynthesis saturation, and the compensation irradiance (E_c), that is, where the O_2 produced via photosynthesis equals the respiratory demands, using the following equations (e.g. Lichtenberg and Kühl 2015):

$$(3)\ E_k = \frac{P_{max}}{\alpha}$$

$$(4)\ E_c = \frac{P_{max} log_{10}(\frac{R}{P_{max}}+1)}{-\alpha}$$

Sample collection, RNA extraction and cDNA synthesis

Four biological replicate samples of *Z. muelleri* were randomly collected for each time point (0 and 24 h) and for each treatment (control and low O_2). Samples included above-ground tissue (i.e. leaf biomass) only, as this part of the plant is likely to respond more immediately, being photosynthetically active and in direct contact with molecular O_2 in the water-column.

Samples were packed in aluminium foil envelopes and snap-frozen directly in liquid nitrogen. Samples were stored at -80°C for 15 days prior to further RNA extraction and RT-qPCR analysis. Briefly, for each sample, ~70 mg of freeze-dried leaf biomass was grounded into powder using a mortar and pestle in liquid nitrogen. RNA was then extracted using the PureLink RNA Mini Kit (Ambion) following manufacturer's instructions. Column purification DNAse digestion was carried out using PureLink DNase Set (Ambion) following the manufacturer's instructions. The RNA quantity and quality was assessed using a spectrophotometer (NanoDrop 2000) and absorbance at 260/280 nm. Good quality RNA samples were stored at -80°C for further RT-qPCR experiments. A total of 500 ng of good quality RNA was used for each sample for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instructions. The resulting cDNA samples were diluted 1:20 for use in RT-qPCR analysis.

Primer design

The present study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (Bustin et al. 2009). In this section, we indicate the essential information, *sensu* Bustin et al. (2009), required to allow reliable interpretation of the corresponding RT-qPCR results.

In-depth analysis of *Zostera muelleri* Transcriptomics Database (Hayward et al, in prep), revealed transcripts encoding proteins with high similarities to the domains of PEPC and CA proteins already identified in the seagrass *Zostera marina* (Olsen et al. 2016). It is interesting to note that the genome of *Z. muelleri*, which was not available at the time of this study, has been published since (Lee et al. 2016). The functional domains of three of these sequences, coding for PEPC1 (KMZ56135), PEPC2 (KMZ58048) and □-CA (KMZ56166) respectively, were used as a template to design sequence-specific primers for RT-qPCR using the software, Primer3 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) with default settings. The

sizes of the resulting amplicons were kept from 79 to 195 bp (Table 1) ensuring similar PCR efficiencies and facilitating cross comparison of assays. The specificity of each selected primer pair was observed by PCR amplification as single bands at the expected size resolved via agarose gel electrophoresis.

Reverse Transcription Quantitative Real Time - PCR and Gene Expression Analysis

SYBR green PCR master mix (Warrington, Cheshire, UK) was used for RT-qPCR assays in 96-wells plates in a Step One Plus™ Real-Time PCR System (Applied Biosystems, USA). PCR conditions were: initial denaturation of 10 min at 95°C, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s and 68°C for 30 s. A dissociation step was included at the end: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The final reaction volume was 10 µL, including 0.8 µL of primers (Table 1) and all reactions were conducted in technical triplicates. The RTqPCR efficiency for each gene and each treatment was determined from a cDNA dilution gradient of 27, 9, 3 and 1 ng and a linear regression model (Pfaffl 2001). The corresponding RT-qPCR efficiencies were calculated according to the equation below (Radonić et al. 2004):

$$(5)\ PCR\ efficiency = (10^{[-\frac{1}{slope}]} - 1) \times 100$$

All the RT-qPCR efficiencies obtained with the different primers were between 96-104%, with a calibration coefficients >0.969 (Table 1, see Supplementary Figure S4). A no template control, as well as a no reverse transcription control was generated for each gene and each treatment to ensure that the PCR reactions were free of DNA contamination.

Data from RT-qPCR was analysed using the Step One Plus™ Software (Ver. 2.3; Applied Biosystems). Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold in the exponential phase of the RT-qPCR reaction. The

cycle threshold (C_T) was set at 0.03 for all genes. To quantify changes in target genes expression, C_T were imported then transformed into quantities using corresponding RT-qPCR efficiency to obtain Normalized Relative Quantities.

Selection of reference genes

In order to select the best reference genes for the experimental conditions, expression stability was analysed using NormFinder (Andersen et al. 2004). The corresponding C_T values were used directly in the software package NormFinder (Andersen et al. 2004) to rank and select the most stable reference genes. Candidate reference genes and corresponding primers used in this study were identified previously (Schliep et al. 2015). Because these candidates reference genes were initially validated under low light stress conditions, we used NormFinder to measure their stability value during low O_2 exposure (i.e. direct measure for the estimated expression variation) as previously described by (Andersen et al. 2004). We also ran complementary analysis using a second software (GeNorm, Vandesompele et al. 2002) which led to similar results as for NormFinder. According to Normfinder, the most stable genes under our experimental conditions were GADPH, Actin and S4 and the best combination of two reference genes: S4 and GADPH (see Supplementary Figure S5) was then used to normalize target gene expression profile in *Z. muelleri* under low O_2.

Statistical analyses

Statistical analyses were performed using a Repeated Measures Analyses of Variance with PERMANOVA+ software in PRIMER v6 (Anderson et al. 2008). The analyses tested the null hypothesis that there is no difference in the α, P_{max}, R, E_k and E_c values derived from the fitted P-I curves of the control and low O_2 treated leaves. The RT qPCR data was analysed in the same way to test the null hypothesis that there is no difference in the normalized relative quantities of PEPC1, PEPC2 and γ CA in control and low O_2 treated plants. We randomized

our sampling within each treatment to minimize lack of independence and to separate the two O_2 levels from other potential effects originating from containers location on the table. Throughout this paper, values given for microsensor data are the mean of 3 biological replicates, while RT-qPCR data are the mean of 4 biological replicates, including technical triplicates. Results were considered significant at 5%.

RESULTS

Rates of net photosynthesis and P-I curves

The vertical O_2 concentration micro-profiles showed a ~0.02 cm thick DBL at the leaf surface of *Z. muelleri* at all irradiances tested in both the control and low O_2 conditions (Figure 1). The average O_2 concentration at the leaf tissue surface of the control plants increased from 203 to 352 µmol O_2 L^{-1} as a response to an increasing incident irradiance from 0 to 100 µmol photons m^{-2} s^{-1}, as compared to an increase from 3.6 to 187 µmol O_2 L^{-1} in the low O_2-treated plants. This translated to statistically different O_2 flux values between the control and low O_2 treated plants at incident photon irradiances of 0, 25 and 50 µmol photons m^{-2} s^{-1} (Permutational t-test, $t4$= 4.7575, P= 0.0026; $t4$= 12.526, P= 0.0002; $t4$= 5.4299, P= 0.0032 respectively, Figure 2,). Comparison between the dark respiration rates (R) and the initial slope of the P-I curve (α), which gives an indication of photosynthetic activity, of the control and low O_2 treated plants also indicated statistical difference (Permutation t-test between control and low O_2 treated plants for R and α; $t4$= 6.8879, P=0.0018; $t4$= 5.2964, P= 0.0064 respectively, Table 2); with a decrease in R but an increase in α.

In contrast, between incident irradiances of 200 to 700 µmol photons m^{-2} s^{-1}, there was only a slight increase of ~2 µmol O_2 L^{-1} in the control plants, from 386 to 388 µmol O_2 L^{-1}, and an increase of ~50 µmol O_2 L^{-1} in the low O_2 treated plants from 194 to 246 µmol O_2 L^{-1}. This translated to net oxygen flux values that were not statistically different among the 200, 500 and 700 µmol photons m^{-2} s^{-1} irradiance treatments (Permutational t-test, $t4$= 2.2527, P= 0.088; $t4$= 0.65804, P= 0.5314; $t4$= 1.147, P= 0.306 respectively, Figure 2). The calculated P_{max} values in the control and low O_2 treated plants, derived from the fitted P-I curves, were also not statistically different (Permutational t-test, $t4$= 0.2956, P= 0.7974, Table 2).

Calculations of the E_k of *Z. muelleri* plants, in the control and low O_2 conditions, yielded an average saturation irradiance of ~60 and 51 µmol photons m^{-2} s^{-1} respectively (Table 2), values that are not statistically different (Permutational t-test, $t4$= 1.2571, P= 0.2892, Table 2); however, there was a statistically significant decrease in the E_c between the control plants and low O_2 treated plants (Student's t-test, $t4$= 6.5624, P= 0.0012, Table 2) which were calculated to be at incident irradiances of ~5.19 and 0.71 µmol photons m^{-2} s^{-1}, respectively (Table 2).

Expression levels of target genes

Among the 7 candidate reference genes tested, the best combination of two reference genes included S4 and GADPH gene (M=0.185; see Supplementary Figure S5). These two reference genes were then used to evaluate target gene expression profile in *Z. muelleri* under low O_2. The relative quantification demonstrated a significant down-regulation of the PEPC-1 and γ-CA genes for seagrass incubated in low O_2 when compared to control (Figure 3). Specifically, there was a ~2.2 fold decrease in PEPC-1 gene expression in low O_2 treated samples relative to control (Permutational t-test, $t6$= 2.9916, P= 0.0188, Figure 3). Similarly, a ~2.8 fold decrease was observed in γ-CA (Permutational t-test, $t6$= 3.3414, P= 0.0072: Figure 3). No statistical difference was observed in the expression level of the PEPC-2 gene (Permutational t-test, $t6$, P= 0.0664, Figure 3), although a decreasing trend was observed in low O_2 treated samples when compared to the controls.

DISCUSSION

Photosynthetic parameters

We observed that by lowering the O_2 concentration in the water-column, the net photosynthetic rates of *Zostera muelleri* were enhanced in the light limited region of the P-I curve (Figure 2). Further, we confirmed that these results were not due to impacts on the health of the *Z. muelleri*

photosystems as shown by the Fv/Fm which remained ≥0.7 for the duration of the experimental period (as measured via PAM-fluorometry, see Supplementary

Figure S6). This shows that *Z. muelleri* has a higher photosynthetic activity with an increased CO_2:O_2 ratio; conditions which potentially could lead to a decreased oxygenase activity of RuBisCO and decreased flux through the PCOC, while increasing carboxylation. Similar studies involving other marine angiosperms such as *Cymodocea rotundata*, *Zostera marina* and *Ruppia maritima* support our findings since the net photosynthetic rate in these plants also increased in response to reduced ambient O_2 concentrations (Black et al. 1976; Downton et al. 1976; Beer et al. 2002; Buapet et al. 2013) whereas low O_2 conditions had no effect on the gross photosynthetic rate of a green alga *Ulva intestinalis* due to suppressed photorespiration (Drechsler and Beer 1991; Beer et al. 2000) via the maintenance of a carbon concentrating mechanism (Björk et al. 1993; Larsson et al. 1997). In addition, within the lacunae of seagrass leaves in light conditions, Carlson et al. (1988) and Roberts and Moriarty (1987) found that O_2 accounted for 38% of the gas within these lacunae, leading to the hypothesis that seagrass leaves may possess a mechanism to minimise photorespiration by inhibiting the accumulation of intracellular O_2. These data show the photosynthetic behaviour of *Z. muelleri* is typical of a plant with C_3 biochemistry and physiology in low O_2 conditions.

We also found a statistically significant decrease in the dark respiration and the subsequent compensation irradiance of the low O_2 treated leaves (Table 2), which was not so surprising owing to the low ambient O_2 availability. A similar effect has been observed in the dark respiration of *Z. marina* when exposed to low O_2 conditions (Buapet et al. 2013). This is believed to be the result of the reduced O_2 availability in the surrounding environment and since O_2 transport to the lacunae ceases after 15 to 30 mins following the onset of darkness (Smith et al. 1984) and the O_2 concentration of the air in the lacunae drops down to <1% of the air around the leaf (Carlson et al. 1988), respiration is decreased. Hence, respiration is an important

factor to take into account when interpreting photosynthetic activity and efficiency responses to changing environmental conditions. Indeed, the effect of low O_2 levels on net photosynthetic rates seen in this study could be solely due to a decreased rate of respiration, particularly as the only statistically significant differences between control and low O_2 treatments were in the 0, 25 and 50 µmol photons m^{-2} s^{-1} irradiances where respiration strongly affects the rate of net photosynthesis, while no statistical difference is seen at the higher irradiances where photorespiration would be expected to have stronger effects (Beardall et al. 2003).

While there appears to be a slight increasing trend in the rate of photosynthesis in the light saturated section of the P-I curve, there was no statistical difference in the saturating irradiance and maximum net photosynthetic rate between the low O_2 treated and control leaf fragments (Figure 2; Table 2). As the pH (and subsequently dissolved inorganic carbon; DIC) was maintained at 8.16 throughout the experiment, our results suggest that the growth of *Z. muelleri* was C-limited and this is consistent with previous findings on other seagrass species (Björk et al. 1997; Zimmerman et al. 1997). With regards to the DIC in seawater, speciation depends on the salinity and temperature, but the main form present at pH 8.16 is HCO_3^- (Pierrot et al. 2006), and HCO_3^- is expected to be the major inorganic C source for photosynthesis in seagrasses. Additional experimentation have shown seagrass to be capable

of utilizing HCO_3^- by means other than uncatalysed conversion of HCO_3^- to CO_2 in the DBL

(Larkum et al. 2017), such as *Halophila stipulacea*, *Thalassodendron ciliatum*, *Halodule uninervis* and *Syringodium isoetifolium* (Beer et al. 1977; Koch et al. 2013; Borum et al.

2016), however the exact method of HCO_3^- uptake remains unclear (Larkum et al. 2017). Moreover, at high photon irradiances O_2 produced via photosynthesis results in similar O_2 microclimates in and around leaves within both treatments, owing to an internal and external build-up of O_2 as a result of the leaf DBLs impeding gas exchange with the surrounding water column (Brodersen et al. 2015). This may therefore explain the similar maximum net

photosynthesis rates measured in the low O_2 and control treatment at photon irradiances ≥100 μmol photons m^{-2} s^{-1} (Fig. 1-2; Table 2). One further point that should not be overlooked is that seagrasses, and many submerged freshwater flowering plants, have photosynthesis almost entirely confined to the epidermis (Larkum et al. 2017). How this anatomical feature affects photosynthesis has been little explored, but its presence in a group of fairly diverse organisms that span several families suggests that it may be important and may affect photosynthesis. Therefore before accepting that seagrasses possess a C_4 metabolism, other explanations should be sought and in this search, gene expression is an important tool.

Gene expression

Within the transcriptome of *Z. muelleri*, we discovered the presence of two different isoforms of PEPC; PEPC-1 and PEPC-2. Molecular differences coupled with differences in phylogenetic relations and gene structure between the two isoforms (Sánchez and Cejudo 2003) have suggested functional differences between the different isoforms in terrestrial plants. However the operation of these isoforms remains to be explored in marine angiosperms. When *Z. muelleri* plants were exposed to low O_2 conditions, we found a significant 2.2-fold decrease in PEPC-1 gene expression (Figure 3). In the terrestrial plant *Arabidopsis*, suppression of an isoform of PEPC via artificial microRNA (amiRNA) impaired root elongation and improved salt tolerance via increasing total PEPC activity (Wang et al.

2012). Little is known about the functionality of this isoform, while we recommend examining the effect of down regulation of PEPC-1 in *Z. muelleri*, the technique for genetic manipulation has not been established in *Z. muelleri* so far. In addition, although there was no statistical difference found in the expression of PEPC-2 in this study (Figure 3); there was a decline in response to low O_2 conditions. As mentioned in the introduction, although PEPC activity has been widely invoked as evidence of C_4 metabolism in aquatic autotrophs, it is also used to feed anaplerotic pathways that produce essential growth compounds such as amino acids (Aubry et

al. 2011). Therefore, it is possible that the down- regulation of PEPC under low O_2 reflects a decreased rate of Krebs cycle throughput however, as we only investigated the expression levels of PEPC as opposed to the activity level of the enzyme in response to low O_2, it is important that the implications of these results are not overly extrapolated. Overall, our results indicate that, when exposed to low water-column O_2, *Z. muelleri* plants (i) increase their photosynthetic activity, a characteristic of C_3 plant photosynthesis and (ii) down-regulate genes coding for PEPC, suggesting that these low O_2 conditions yield lower energy costs of photosynthesis.

HCO_3^- can be utilised through extracellular dehydration via CA (Millhouse and Strother 1986a; Beer and Rehnberg 1997; Invers et al. 2001), although this alone does not constitute a CCM (Larkum et al. 2017). In the case of *Z. muelleri*, inhibition of CA activity via acetazolamide (a membrane-impermeant CA inhibitor, so only inhibiting extracellular CA) has been shown to inhibit photosynthetic use of HCO_3^- (Millhouse and Strother 1986b; Koch et al. 2013; Borum et al. 2016). Of the 5 known independently evolved classes of CA (α, β, γ, δ and ζ; Tripp et al. 2001) and the recently described η class (Del Prete et al. 2014), we investigated the expression levels of γ-CA. For this, γ-CA was selected as the sub-complexes are contained in the respiratory complex 1 (NADH:ubiquinone oxidoreductase) of plants and algae and in the mitochondrial respiratory electron transport chain, with sub-complexes serving as the entry point of electrons, potentially playing a role in photorespiration, probably as a HCO_3^- transporter rather than as a normal CA (Braun and Zabaleta 2007; Martin et al. 2009). In agreement with photosynthetic response and regulation in PEPC genes, the significant down regulation of γ-CA genes (2.8-fold decrease: Figure 3) observed in *Z. muelleri* plants exposed to low O_2 suggests that this enzyme might be more critical for photosynthesis under ambient O_2 levels (i.e. lower DIC:O_2) then under low O_2 levels.

In the freshwater aquatic monocot *Hydrilla verticillata*, C_4-type photosynthesis is induced in C_3-type photosynthesising leaves under warmer temperatures, limited CO_2, increased O_2 and high photon irradiances (Bowes and Salvucci 1989). In this way, aquatic plants could have the capacity to acclimate to a changing climate, therefore highlighting the need to better understand these mechanisms especially in keystone seagrass species such as *Z. muelleri*.

Experimental manipulation of photosynthesis in aquatic organism can be complex as several factors need to be carefully considered. Firstly, avoiding pseudo-replication at the chamber/aquarium level is certainly desirable. In this respect, we recognize the limitations of our experimental design as each of the treatments (i.e. control and low O_2) had one sump (100L plastic bin) feeding plastic container replicates, which is not ideal for full replication. However, we have used 4 container replicates for each treatment and we have randomized our sampling within each treatment to ameliorate some of the risks (Hurlbert 1984) and to separate the two O_2 levels from other potential effects originating from container replicates location on the table. This type of design is commonly used in experiments simulating ocean acidification (Sinutok et al. 2011; Sinutok et al. 2012; Sinutok et al. 2014). Secondly, it is also important to make sure that the experimental procedure does not affect multiple components of water chemistry, particularly inorganic carbon levels within different treatment tanks. While we did not perform any alkalinity measurements during the experiment, we can be confident that the various forms of inorganic carbon were in equilibrium during our experiment for the following reasons:

i) The O_2 level in both the treatment and control tanks was stable throughout the experimental period (Supplementary Figure S2).

ii) 99.9% pure CO_2 gas was used in the treatment tank to control the pH which was done via a pH controller connected to a pH/CO_2 controller (7074/2, TUNZE Aquarientechnik

GmbH, Germany), to maintain the same pH of 8.16 in both tanks (Supplementary Figure S3). This calibrated pH probe constantly measures the pH of the aquaria whereupon as pH starts to increase due to the flushing of nitrogen gas displacing the dissolved CO_2, subsequently reducing the concentration of HCO_3^-, the controller immediately switches on the CO_2 gas to stop the deviation and return the pH back to the experimental level (display accuracy of +/- 0.01 pH). Since this process occurs continuously, the pH deviates between 0.01-0.05 throughout the experimental period and these deviations were rectified automatically over several seconds.

iii) The tanks were kept in a temperature controlled room and salinity was kept constant throughout the experimental period.

iv) The time required for the various forms of inorganic carbon to reach equilibrium in seawater is at most 10s (see Zeebe and Wolf-Gladrow 2001).

This is the first study to combine microsensors and gene expression analyses to investigate responses to low O_2 in *Z. muelleri* and further studies with more sophisticated experimental set up are clearly needed to give more informative results.

Conclusion

Ambiguous metabolic properties such as the ability to use HCO_3^- and a C_4-type photosynthetic quantum efficiency have led to some seagrass species being classified as C_3C_4 intermediate plants (Beer et al. 1980; Beer and Wetzel 1981; Bowes and Salvucci 1989).

We suggest that the photosynthetic classification of *Z. muelleri* should also be carefully considered, as our results indicate that (i) ambient levels of O_2 affect the photophysiology of this seagrass, a characteristic of C_3 plants, and (ii) low O_2 levels induce the down-regulation of PEPC and □-CA genes. While regulation of these genes might not be strictly associated with a C_4 biochemistry, our data suggests that when the conditions are favourable for the

carboxylation reaction of RuBisCO, *Z. muelleri* down-regulates its CCM(s), thus altering its photophysiology. Future work involving the precise measurement of photorespiration and respiration is needed to show how photorespiration and respiration affect the photosynthetic response of seagrass to low O_2. Furthermore, localization and activity of PEPCs, γ-CA and Rubisco and measurements of short-term (2-5 seconds) inorganic ^{14}C labelling products, is needed to show some type of compartmentalization between initial HCO_3^- incorporation via PEPC and the final fixation of CO_2 via Rubisco, thereby supporting or not the role these enzymes play in the physiology of seagrasses. This research is not only needed to enable development of testable hypotheses to better direct future research, but also to improve the management and protection of these environmentally important marine angiosperms.

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Table 1. Reference genes and target genes investigated in *Zostera muelleri* by using RT-qPCR. Accession numbers of the closest sequence matches available online in the data repository for *Zostera marina* EST (**{** HYPERLINK "http://drzompo.uni-muenster.de/" \h **}{** HYPERLINK "http://drzompo.uni-muenster.de/" \h **}{** HYPERLINK "http://drzompo.uni-muenster.de/" \h **}** primers sequences, amplicon length, melting temperature, geometric mean of cycle threshold (CT) and RT-qPCR efficiency are indicated. GADPH: Glyceraldehyde 3-phosphate dehydrogenase; EloF: Translation initiation factor 1 subunit beta; Calmodulin; TubB: Tubulin beta-1 chain; Actin; PolyA: Poly(A) RNA polymerase; S4: 30S ribosomal protein S4; PEPC-1: Phosphoenolpyruvate carboxylase – isoform 1; PEPC-2: Phosphoenolpyruvate carboxylase – isoform 2 and γ-CA: γ Carbonic anhydrase.

Name	Accession number	Forward primer	Reverse primer	Length (bp)	T_m (%)	C_T	Efficiency
GAPDH	Zoma_C_c6252	CGGTTACTGTAGCCCCACTC	CAAAGGCTGGGATTGGTTTA	79	59.9	25	88
EloF	Zoma_C_c59090	AAGCAAAGGCGTCACTTGAT	TCTGCTGCCTTCTTCTCCTC	82	59.9	24	104
Calmodulin	Zoma_B_i07192	ATCCATCCTGGTCTTTGTCG	CACTGTGATCCACTCGTTGG	197	60.1	23	114
TubB	Zoma_Contig120	GGACAAATCTTCCGTCCAGA	TCCAGATCCAGTTCCACCTC	195	60	24	88
Actin	Zoma_ZMF02257	TAAGGTCGTTGCTCCTCCTG	ACTCTGCCTTTGCAATCCAC	104	60.4	26	110
PolyA	Zoma_C_c36619	GCTGCTCGTTCAAATTCCTC	ATGACCGCCATTTAATCTGC	112	59.9	29	93
S4	Zoma_Contig219	ATGGTCTGACAGAGCGACAA	TGTTATCCAAACGCATCTCG	108	59.7	29	114
PEPC-1	KMZ56135	AGGCAAAATTCGGACTTCCT	GAGGACGCAGTGTTGACAGA	84	60.1	29	99
PEPC-2	KMZ58048	TGGCTGTTGTAGCCACTGAG	TCTGTCTCTGGTGTGGCAAG	91	60	28	97
γ-CA	KMZ56166	AGGTCATGGTGCTGTCCTTC	CAGCAACCATTCCGTTCTTT	110	60.1	28	104

Table 2. The initial slope of the P-I curve in the light-limiting phase (α), maximum net photosynthetic rate (P_{max}), dark respiration rate (R), compensation irradiance (E_c) and minimum saturating irradiance (E_k) in *Zostera muelleri* leaves exposed to water-column O_2 levels of ~231

µmol O_2 L^{-1} (control) and ~8 µmol O_2 L^{-1} (low O_2). Values are given as a mean ±SEM (n=3); with their corresponding P values (Permutation ttest), where * indicate significant difference between treatments on a 5% level.

	α	**P_{max} (nmol O_2 cm^{-2} h^{-1})**	**R (nmol O_2 cm^{-2} h^{-1})**	**E_k (µmol photons m^{-2} s^{-1})**	**E_c (µmol photons m^{-2} s^{-1})**
Control O_2	10.95±0.4	668.14±80.3	-117.09±12.5	60.82±6.0	5.19±0.8
Low O_2	13.34±0.1	687.85±49.3	-21.42±3.9	51.66±4.2	0.71±0.1
P	0.0064*	0.7974	0.0018*	0.2892	0.0012*

874 Figure Legends

875

876 **Fig. 1** Vertical O_2 concentration micro-profiles measured at the surface of *Zostera muelleri*
877 leaves. Filled circles and solid lines represent data from the control leaves (i.e. leaves exposed
878 to a water-column O_2 concentration of ~231 µmol O_2 L^{-1}), while open circles and dashed
879 lines represent data from leaves exposed to low O_2 conditions (i.e. ~8 µmol O_2 L^{-1}). Incident
880 photon irradiances are indicated by the figure legend (i.e. 0, 25, 50, 100, 200, 500 and 700
881 µmol photons m^{-2} s^{-1}). Error bars are ± standard error of the mean (SEM). Y = 0 indicate the
882 leaf tissue surface. n=3

883 **Fig. 2** Net photosynthesis of *Zostera muelleri* leaves at an incident photon irradiance of 0, 25,
884 50, 100, 200, 500 and 700 µmol photons m^{-2} s^{-1}. Data points were fitted with an exponential
885 saturation function (Webb et al., 1974) with an added respiration term, R, to account for
886 respiration (Spilling et al., 2010). Black squares and line represent data of leaves kept in ~231
887 µmol O_2 L^{-1} (i.e. control plants), while open triangles and dashed line represent data of leaves
888 kept in ~8 µmol O_2 L^{-1} (i.e. low O_2 treatment). Error bars are ±SEM; while statistically
889 different values are indicated by * (Permutation t-test, $P< 0.05$). n=3

Fig. 3 Normalized relative quantity (NRQ) of Phosphoenolpyruvate carboxylase (PEPC-1 isoform 1 and PEPC-2 isoform 2) and γ Carbonic anhydrase (γ-CA) in *Zostera muelleri* under control (solid bars) and low O_2 conditions (open bars) relative to the two most stable reference genes: S4 and GADPH. Statistical differences in the mean are indicated with * (Permutation t-test, $P< 0.05$) and error bars are ±SEM. n=4